Branswick Labs.

Highlight of Our Services: the ORAC Assay

1. What does "ORAC" stand for?

"ORAC" is an abbreviation of "Oxygen Radical Absorption Capacity"

2. Who are the original ORAC developers?

The ORAC assay was originally developed by Dr. Guohua Cao of the National Institute of Aging in 1992[1]. In 1996, Dr. Cao joined Dr. Ronald L. Prior's group at Jean Mayer USDA Human Nutrition Research Center on Aging, where Drs. Cao and Prior were instrumental in semi-automating the ORAC assay[2]. Since then, the ORAC assay has been extensively utilized in the field of antioxidant and oxidative stress[3,4,5,6,7].

3. What is the principle of the ORAC assay?

The ORAC assay depends on the free radical damage to a fluorescent probe through the change in its fluorescence intensity. The change of fluorescence intensity is an index of the degree of free radical damage. In the presence of antioxidant, the inhibition of free radical damage by an antioxidant, which is reflected in the protection against the change of probe fluorescence in the ORAC assay, is a measure of its antioxidant capacity against the free radical (Figure 1).

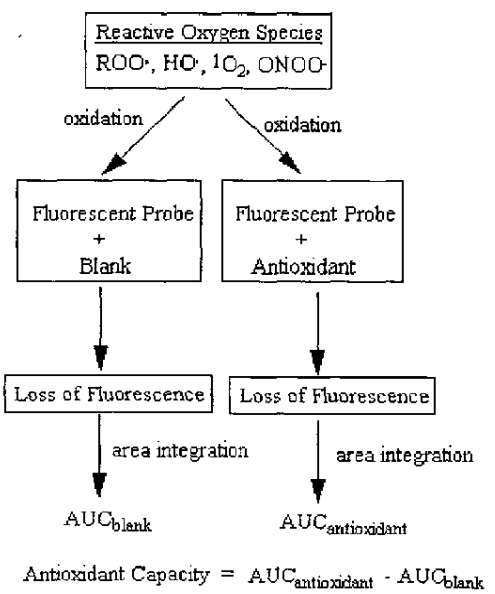

Figure 1. Principle of the ORAC assay

PM3006722769

The uniqueness of the ORAC assay is that the reaction is driven to completion and the quantitation is achieved using "area under the curve" (AUC) (Figure 2).

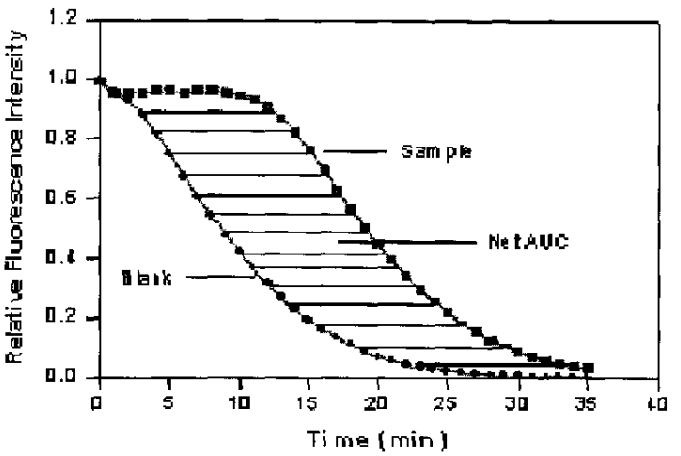

Figure 2. Antioxidant activity of tested sample expressed as the net area under the curve (AUC)

In particular, the AUC technique allows ORAC to combine both inhibition time and inhibition percentage of the free radical damage by the antioxidant into a single quantity (Figure 3).

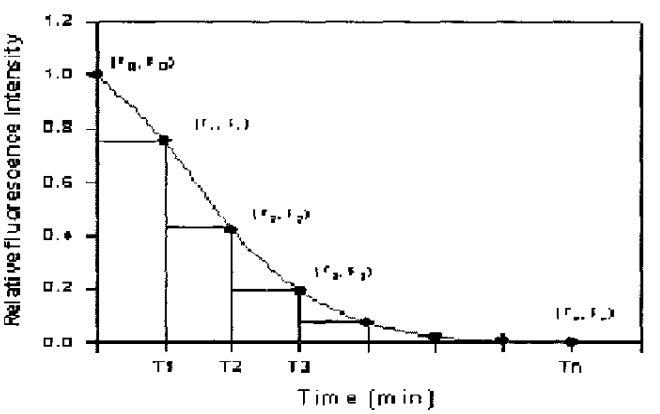

Figure 3. Calculation of ORAC values. AUC=0.5 + F_1/F_0 +F_2/F_0+F_3/F_0+.....+Fn/F_0; Relative ORAC value = [(AUC_{Sample}- AUC_{Blank})/($AUC_{Standard}$- AUC_{Blank})]* (molarity of standard/molarity of sample)

4. What does the ORAC assay actually measure?

The original ORAC is primarily for water-soluble antioxidant activity against the peroxyl radicals. Recently we expanded the ORAC method to fat-soluble antioxidant activity. Soon thereafter, we introduced the hydroxyl radical in the ORAC assay panel.

5. What are the drawbacks of the original ORAC method?

B-phycoerythrin (B-PE), a protein isolated from B-phycoerythrin (B-PE), was the chosen fluorescent probe in the original ORAC method. However, B-PE was found to interact with phenolic compounds that are thought to be main antioxidants derived from plants. The nonspecific protein binding can cause falsely low ORAC value. Therefore it is problematic to use B-PE to evaluate antioxidant activity[8].

6. What improvement on ORAC was made by Brunswick Laboratories?

In collaboration with Dr. Ronald Prior of the USDA, the scientists at BL significantly improved the ORAC assay. Briefly, B-PE was replaced by a stable compound called fluorescein. Since fluorescein is a synthetic fluorescence compound, it will not interact with the tested samples. In addition, unlike the original method the current ORAC method is suitable for both water-soluble and fat-soluble antioxidants. Furthermore, we successfully integrated the hydroxyl radicals into the assay.

6. Do you have literature references about the use of fluorescein for the ORAC assay?

We are the first group who reported the use of fluorescein for the both water-soluble and fat-soluble antioxidants. Please feel free to request a copy of our peer-reviewed papers published in the Journal of Agricultural and Food Chemistry[9,10,11]

7. How many harmful reactive species existing in the body?

There are 6 harmful common reactive oxygen or nitrogen species existing in the body. They are peroxyl radical (ROO•), hydroxyl radical (HO•), hydrogen peroxide (H_2O_2), superoxide ion ($O_2^{-\bullet}$), singlet oxygen ($^1O_2$) and peroxynitrite (OONO-)[12].

8. Does the current ORAC assay measure the total antioxidant activity against all the radicals?

No. The current ORAC assay only measures the antioxidant activity against peroxyl radical and hydroxyl radical. We are in the process to expand the ORAC method to other harmful species.

9. What samples can be tested for ORAC?

Natural products, antioxidant supplements, beverages, pure chemicals, plasma, serum, urine.

10. How are samples prepared prior to shipping?

Sample can be shipped "as is" and no special sample preparation is needed prior to shipping. Biological samples need to be stored in dry ice for overnight shipping.

11. what are the Applications of ORAC

- Natural Foods & Agricultural Produce
- Quality Control of Antioxidant Products
- Clinical Trial of Antioxidant Products (Figure 5)
- Clinical Assessment of Aging

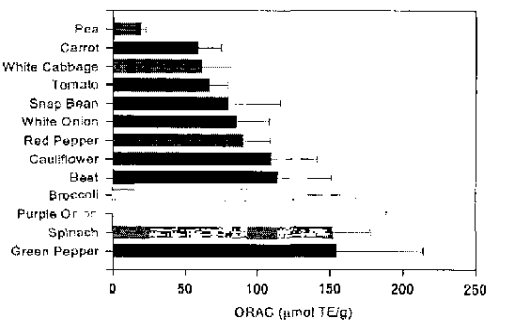

Figure 4. ORAC ranking of vegetables

11. How long does the test take to perform?

One hour for sample preparation, 35 min for measurement.

12. What is the standard used in the ORAC assay?

For peroxyl radical, the standard is Trolox, an analogue of vitamin E; For hydroxyl radical, the standard is gallic acid.

13. What is the unit of the ORAC result?

The ORAC unit is expressed as micro mole standard per gram or Liter.

14. Can I use the ORAC result as a quality control for my product development?

Yes. We can test your product from lot to lot and batch to batch.

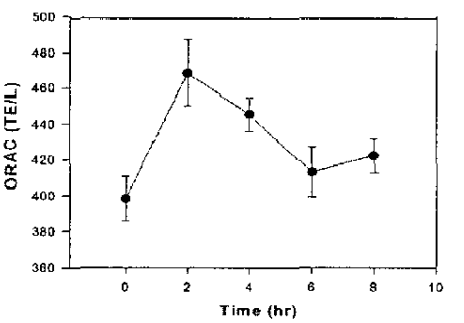

Figure 5 Increased antioxidant capacity of human plasma after intake of a grape juice product

15. Can I use the published ORAC data for comparison purpose?

No. In the middle of the 90's, Cao and coworkers published the ORAC values of some vegetables and fruits[13]. The results primarily demonstrate that the ORAC assay is a valuable tool to evaluate the antioxidant activity. However, due to the limited sample sizes and varieties, the published ORAC values of vegetables and fruits are not comprehensive. Importantly, the most published ORAC results are obtained using the old method, therefore they are not valid any more. Furthermore, the naturally occurring antioxidants actually are the secondary metabolites of natural products, the mother nature has a profound influence on their biosysthesis

pathways. For instance, the geographic locations, weather conditions and the varieties of the species have been determined to influent the antioxidant property chemically and physiologically. We screened over 1000 vegetables last year, the conclusion is that "not every broccoli is created equal" [14].

16. Why the ORAC assay is advantageous over many other methods?

The mechanism of ORAC is based upon the sound chemical principle and the uniqueness of ORAC lies in the AUC quantitation technique. Indeed, many other methods have been developed for antioxidant activity, such as TEAC (Trolox Equivalent Antioxidant Capacity)[15], TOSC (Total Oxyradical Scavenging Capacity)[16], FRAP (Ferric Reducing Antioxidant Power)[17], and DPPH method[18]. However, the fatal drawback of these methods is neither no oxygen radial involved or lack of complete quantitation technique. Therefore, only ORAC provides antioxidant activity mechanistically and physiologically.

16. What are the potential interferences/ pitfalls of this assay?

The improved ORAC assay has been validated through specificity, linearity, reproducibility and sensitivity. The validation results indicated that the ORAC is robust. No interferences have been apparent.

17. Can I use HPLC method to quantify antioxidant activity?

No. The HPLC method only measures the quantity of a specific compound instead of antioxidant activity.

18. What instrument is used for the ORAC assay?

We are using the 96-well fluorescence microplate reader coupled with the 8-channel liquid handling system. This full automation completely limits humane errors during sample preparation and measurement[19]. Therefore, our results are highly reproducible.

19. Who are the competitors for the ORAC testing service?

Brunswick Laboratories is the inventor of the improved and automated ORAC assay (US patent pending). Indeed, there are couple of commercial labs who are providing the ORAC service, however, all of them are still using the invalidated B-PE based ORAC method.

Literature Cited

[1] Cao, G. H.; Alessio, H. M.; Cutler, R. G. Oxygen-Radical Absorbency Capacity Assay for Antioxidants. *Free Radic. Biol. Med.* **1993**, *14*, 303-311.

[2] Cao, G.; Verdon, C. P.; Wu, A. H. B.; Wang, H.; Prior, R. L. Automated-Assay of Oxygen Radical Absorbency Capacity with the Cobas Fara-Ii. *Clin. Chem.* **1995**, *41*, 1738-1744.

[3] Ehlenfeldt, M. K.; Prior, R. L. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. *J. Agric. Food Chem.* **2001**, *49*, 2222-2227

[4] Cao, G.; Sanchez-Moreno, C.; Prior, R. L. Procyanidins, anthocyanins and antioxidant capacity in wines. *Faseb J.* **2000**, *14*, A564-A564.

[5]Cao, G. H.; Shukitt-Hale, B.; Bickford, P. C.; Joseph, J. A.; McEwen, J.; Prior, R. L. Hyperoxia-induced changes in antioxidant capacity and the effect of dietary antioxidants. *J. Appl. Physiol.* **1999**, *86*, 1817-1822.

[6] Cao, G. H.; Booth, S. L.; Sadowski, J. A.; Prior, R. L. Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. *Am. J. Clin. Nutr.* **1998**, *68*, 1081-1087.

[7] Cao, G. H.; Prior, R. L.; Cutler, R. G.; Yu, B. P. Effect of dietary restriction on serum antioxidant capacity in rats. *Arch. Gerontol. Geriatr.* **1997**, *25*, 245-253.

[8] Cao, G. H.; Shukitt-Hale, B.; Bickford, P. C.; Joseph, J. A.; McEwen, J.; Prior, R. L. Hyperoxia-induced changes in antioxidant capacity and the effect of dietary antioxidants. *J. Appl. Physiol.* **1999**, *86*, 1817-1822.

[9] Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical oxygen assay using fluorescein as the fluorescent Probe. *J. Agric. Food Chem.* **2001**, *49, 4619-4626*

[10] Huang, D., Ou, B., Hampsch-Woodille, M. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated beta-cyclodextrin as the solubility enhancer. *J. Agric. Food Chem.* **2002**, *50,1815-1821.*

[11] Huang, D., Ou, B., Hampsch-Woodille, M., Prior, RL Measurement of hydroxyl radical prevention capacity. *J. of Agric. and Food Chem.* **2002**, AAPS.

[12] Halliwell, B. Reactive Oxygen Species in Living Systems - Source, Biochemistry, and Role in Human-Disease. *Am. J. Med.* **1991**, *91*, S14-S22.

[13] Cao, G. H.; Sofic, E.; Prior, R. L. Antioxidant capacity of tea and common vegetables. *J. Agric. Food Chem.* **1996**, *44*, 3426-3431.

[14] Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J., Deemer, E.. Analysis of antioxidant activities of common vegetables employing Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP) assays: A comparative study. *J. Agric.Food Chem.* **2002**, ASAP.

[15] Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231-1237.

[16] Winston, G. W.; Regoli, F.; Dugas, A. J.; Fong, J. H.; Blanchard, K. A. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. *Free Radic. Biol. Med.* **1998**, *24*, 480-493.

[17] Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70-76.

[18] Hirano R., Sasamoto W., Matsumoto A. Antioxidant ability of various flavonoids against DPPH radicals and LDL oxidation. *J NUTR SCI VITAMINOL.* **2001**, 47, 357-362.

[19] Huang, D., Ou, B., Hampsch-Woodille, M., Flanagan, J. High Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using the Multi-Channel Liquid Handling System Coupled with the Microplate Fluorescence Reader in 96-well Format. *J. Agric.Food Chem.* **2002,** *in press.*